PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

United States Patent and Trademark Office
(Box PCT)
Crystal Plaza 2
Washington, DC 20231
ÉTATS-UNIS D'AMÉRIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 10 May 1999 (10.05.99)	
International application No. PCT/SE98/01638	**Applicant's or agent's file reference** 47367-53046
International filing date (day/month/year) 15 September 1998 (15.09.98)	**Priority date** (day/month/year) 15 September 1997 (15.09.97)
Applicant MATTIASSON, Bo et al	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International Preliminary Examining Authority on:

01 April 1999 (01.04.99)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland**	Authorized officer Nicola Wolff
Facsimile No.: (41-22) 740.14.35	Telephone No.: (41-22) 338.83.38

PATENT COOPERATION TREATY

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INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
47367-53046		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/SE98/01638	15/09/1998	15/09/1997
International Patent Classification (IPC) or national classification and IPC G01N33/543		
Applicant VLAAMSE INSTELLING VOOR TECHNOLOGISCH ONDERZOEK		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 4 sheets, including this cover sheet.

   ☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which hav been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 5 sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☐ Certain documents cited
   - VII ☐ Certain defects in the international application
   - VIII ☐ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
01/04/1999	20.09.99
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer Hinchliffe, P Telephone No. +49 89 2399 8431

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/SE98/01638

I. Basis of the report

1. This report has been drawn on the basis of (*substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments.*):

Description, pages:

1,4-24	as originally filed			
2,3	as received on	06/09/1999	with letter of	02/09/1999

Claims, No.:

1-10	as received on	06/09/1999	with letter of	02/09/1999

Drawings, sheets:

1/9-9/9	as originally filed

2. The amendments have resulted in the cancellation of:

- ☐ the description, pages:
- ☐ the claims, Nos.:
- ☐ the drawings, sheets:

3. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

4. Additional observations, if necessary:

V. Reasoned statement under Article 35(2) with regard t novelty, inventiv step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	1-10
	No:	Claims	
Inventive step (IS)	Yes:	Claims	1-10
	No:	Claims	
Industrial applicability (IA)	Yes:	Claims	1-10
	No:	Claims	

2. Citations and explanations

see separate sheet

SECTION V

1. The subject matter of claims 1-10 refers to a method for producing a metal ion specific electrode, products and uses thereof. The subject matter is different to the methods disclosed in the prior art documents cited in the ISR. The closest prior art is considered to be Rojas et al, J.Am.Chem.Soc., 1995, vol.117, p.336-343. This document discloses a cyclodextrin based metal ion sensitive electrode in which the imperfect cyclodextrin monolayer is "filled in" after formation. The present method differs insofar as the first monolayer is formed without the ion selective agent being present. The ion selective agent is added in the next step followed by a final "filling in" step. The results of this method appear to produce a sensor which is several orders of magnitude higher in sensitivity than the closest prior art sensor. Such an improvement was not suggested or hinted at in any of the documents cited in the ISR.
   Consequently the subject matter of the claims can be considered to fulfill the requirements of both Articles 33(2) and 33(3) PCT.

Claims

1. A method for producing a metal ion-specific capacity affinity sensor suitable for determining the presence of a certain heavy metal ion by capacitance measurement, comprising the steps of:

a) providing a piece of a noble metal, where said piece optionally can be a rod, or alternatively a piece of insulating material such as glass, silicon or quartz, on which a noble metal is sputtered or printed;

b) providing a first SAM-forming molecule comprising a coupling group;

c) contacting the piece in step a) with the first SAM-forming molecule in step b), thereby obtaining a self-assembling monolayer on said noble metal surface;

d) contacting said self-assembling monolayer on said noble metal piece with a molecule specifically binding said heavy metal ion, thereby coupling said molecule to the self-assembling monolayer;

e) contacting the piece obtained in step d) with a second SAM-forming molecule, thereby obtaining a noble metal surface that is at least 90%, preferably at least 95%, more preferably at least 97% and most preferably at least 99% covered with a self-assembling monolayer.

2. A method according to claim 1, characterized in that the coupling reaction in step d) is carried out in presence of PEGDGE.

3. A method according to claim 1, characterized in that the piece is exposed to a solution containing a crosslinking substance such as glutaraldehyde prior to step d).

4. A method according to claim 1, characterized in that the first SAM-forming molecule is D/L-thioctic acid, and in that said D/L-thioctic acid is activated with 1-(3-dimethylaminopropyl)-3-ethyl-carbodiimide before step d) is carried out.

Replaced by Article 34

5. A method according to claim 1, characterized in that the second SAM-forming molecule is a thiol comprising 3-25 carbon atoms in a straight saturated chain, and preferably is 1-dodecanethiol.

6. A metal ion-specific capacity affinity sensor comprising a piece of a noble metal, where said piece optionally can be a rod, or alternatively a piece of insulating material such as glass, silicon or quartz, on which a noble metal is sputtered, to which piece groups specifically binding to a certain heavy metal ion of interest have been bound characterized in that said groups specifically binding to said heavy metal ion are bound to a self-assembling monolayer covering at least 90%, preferably at least 95%, more preferably at least 97%, and most preferably at least 99% of the noble metal surface.

7. A metal ion capacity affinity sensor according to claim 6, characterized in that said sensor has been produced by a method according to anyone of claims 1-6.

8. A sensor according to claim 6, characterized in that specifically heavy metal ion-binding groups are selected from the group of proteins having the sequences SEQ.ID.NO.1, SEQ.ID.NO.2, SEQ.ID.NO.3 or SEQ.ID.NO.4, or functional derivatives thereof having equivalent binding characteristics.

9. A method for qualitatively or quantitatively determining the presence of a certain heavy metal ion of interest in a liquid sample, comprising the steps of:

a) providing a sensor according to claim 6, wherein said affinity groups specifically binds to said heavy metal ion of interest;

b) contacting said sensor with a reference liquid not containing said heavy metal ion of interest and determining the capacitance according to per se known methods;

c) contacting said sensor with a sample suspected of containing said heavy metal ion and determining the capacitance according to per se known methods; and

d) calculating the difference between the capacitance of the sample and the capacitance of the reference sample, and optionally calculating the amount of said compound by using prerecorded calibration data.

10. A method according to claim 9 for determining the presence of ions selected from the group of Zn^{2+}, Hg^{2+}, Cd^{2+}, Cu^{2+}, and Pb^{2+}.

11. Use of a sensor according to claim 6 for determining the presence of of ions selected from the group of Zn^{2+}, Hg^{2+}, Cd^{2+}, Cu^{2+} and Pb^{2+}.

engineered to be exclusively selective towards Hg^{2+} [Frantz, B.; O'Hallaran, T. V.; *Biochemistry*, 29, 1990 4747-4751]. A third protein, PbrR (SEQ.ID.NO.3) from the strain Alcaligenes eutrophus CH34 (The strain is deposited BCCM under the accession number LMG P-18077) is selective towards Pb^{2+}. A fourth protein, *Mer*P (SEQ.ID.NO.4), is selective towards Hg^{2+}.It is believed that a large conformational change is taking place when heavy metal ions bind to these proteins. This invention describes a capacitive sensor which can detect these conformational changes directly.

Self-assembled monolayers of thiols, sulfides and disulfides on gold electrodes have been widely studied and long-chain alkanethiols are known to form insulating well-organized structures on gold substrates [Porter, M. D.; Bright, T. B.; Allara, D. L.; Chidsey, C. E. D. *J. Am. Chem. Soc* 1987, *109*, 3559-3568]. The binding formed between the sulphur atom and gold is very strong and the formed self-assembled monolayers (SAM's) are stable in air, water and organic solvents at room temperature [Bain, C. D.; Troughton, E. B.; Tao, Y.-T.; Evall, J.; Whitesides, G. M.; Nuzzo, R. G. *J. Am. Chem. Soc.* 1989, *111*, 321-335]. It has been suggested that micro-contact printing [Mrksich, M.; Whitesides, G. M. *Tibtech* 1995, *13*, 228-235] and photolithography [Bhatia, S. K.; Hickman, J. J.; Ligler, F. S. *J. Am. Chem. Soc.* 1992, *114*, 4432-4433] can be used to pattern surfaces with functionalized self-assembled monolayers for biosensor production with low cost for a diversity of applications, but until now it has not been possible to produce direct affinity sensors with high sensitivity.

Summary of the invention

It has now turned out that unexpectedly good metal ion-specific capacity affinity sensors suitable for determining the presence of a certain heavy metal ion by capacitance measurement, comprising the steps of:

a) providing a piece of a noble metal, where said piece optionally can be a rod, or alternatively a piece of insulating material such as glass, silicon or quartz, on which a noble metal is sputtered or printed;

b) providing a first SAM-forming molecule comprising a coupling group;

c) contacting the piece in step a) with the first SAM-forming molecule in step b), thereby obtaining a self-assembling monolayer on said noble metal surface;

d) contacting said self-assembling monolayer on said noble metal piece with a molecule specifically binding said heavy metal ion, thereby coupling said molecule to the self-assembling monolayer;

e) contacting the piece obtained in step d) with a second SAM-forming molecule, thereby obtaining a noble metal surface that is at least 90%, preferably at least 95%, more preferably at least 97%, and most preferably at least 99% covered with a self-assembling monolayer.

Detailed description of the invention

The detection limits reported in this invention are several orders of magnitude better than those reported previously for electrochemical metal detection methods. The insights behind this invention are that the recognition layer must be thin, well-ordered and it must cover at least 90%, preferably at least 95%, more preferably at least 97%, and most preferably at least 99% of the sensor surface. In a subsequent step, any free spots between the recognition elements are "plugged", i.e. covered with a second self-assembling monolayer-forming molecule, e.g. an alkanethiol comprising 3-25 carbon atoms preferably in a straight chain , after obtaining a self-

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Self-assembled monolayers of thiols, sulfides and disulfides on gold electrodes have been widely studied and long-chain alkanethiols are known to form insulating well-organized structures on gold substrates [Porter, M. D.; Bright, T. B.; Allara, D. L.; Chidsey, C. E. D. *J. Am. Chem. Soc* 1987, *109*, 3559-3568]. The binding formed between the sulphur atom and gold is very strong and the formed self-assembled monolayers (SAM's) are stable in air, water and organic solvents at room temperature [Bain, C. D.; Troughton, E. B.; Tao, Y.-T.; Evall, J.; Whitesides, G. M.; Nuzzo, R. G. *J. Am. Chem. Soc.* 1989, *111*, 321-335]. It has been suggested that micro-contact printing [Mrksich, M.; Whitesides, G. M. *Tibtech* 1995, *13*, 228-235] and photolithography [Bhatia, S. K.; Hickman, J. J.; Ligler, F. S. *J. Am. Chem. Soc.* 1992, *114*, 4432-4433] can be used to pattern surfaces with functionalized self-assembled monolayers for biosensor production with low cost for a diversity of applications, but until now it has not been possible to produce direct affinity sensors with high sensitivity.

Rojas et al., J. Am. Chem. Soc. 1995, 117, 336-343 relates to a capacity affinity sensor for assaying ferrocene, Per-6-thio-ß-cyclodextrin, a compound capable of forming a self-assembling monolayer, is bound to a gold surface. Monolayer defects are covered by treatment with a solution of ferrocene and pentanethiol. Steinberg et al., J. Am. Chem. Soc. 1991, 113, 5176-5182 relates to a capacity affinity sensor wich has been produced by adsorbing 2,2'-thiobis (ethylacetoacetate) or by simultaneously adsorbing 2,2'-thiobis (ethylacetoacetate) and n-pctadecyl mercaptane to a gold surface. The effect of applied potential ion binding is investigated.

Claims

1. A method for producing a metal ion-specific capacity affinity sensor suitable for determining the presence of a certain heavy metal ion by capacitance measurement, comprising the steps of:

a) providing a piece of a noble metal, where said piece optionally can be a rod, or alternatively a piece of insulating material such as glass, silicon or quartz, on which a noble metal is sputtered or printed;

b) providing a first self-assembling monolayer-forming molecule comprising a coupling group;

c) contacting the piece in step a) with the first self-assembling monolayer-forming molecule in step b), thereby obtaining a self-assembling monolayer on said noble metal surface;

d) contacting said self-assembling monolayer on said noble metal piece with a molecule specifically binding said heavy metal ion, thereby coupling said molecule to the self-assembling monolayer;

e) contacting the piece obtained in step d) with a second Self-assembling monolayer-forming molecule, thereby obtaining a noble metal surface that is at least 90%, preferably at least 95%, more preferably at least 97%, and most preferably at least 99% covered with a self-assembling monolayer.

2. A method according to claim 1, characterized in that the coupling reaction in step d) is carried out in presence of PEGDGE.

3. A method according to claim 1, characterized in that the piece is exposed to a solution containing a crosslinking substance such as glutaraldehyde prior to step d).

4. A method according to claim 1, characterized in that the first self-assembling monolayer-forming molecule is D/L-thioctic acid, and in that said D/L-thioctic acid is activated with 1-(3-dimethylaminopropyl)-3-ethyl-carbodiimide before step d) is carried out.

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6. A metal ion-specific capacity affinity sensor comprising a piece of a noble metal, where said piece optionally can be a rod, or alternatively a piece of insulating material such as glass, silicon or quartz, on which a noble metal is sputtered, to which piece groups specifically binding to a certain heavy metal ion of interest have been bound characterized in that said groups specifically binding to said heavy metal ion are bound to a self-assembling monolayer covering at least 90%, preferably at least 95%, more preferably at least 97%, and most preferably at least 99% of the noble metal surface characterized in that said sensor has been produced by a method according to anyone of claims 1-6.

7. A sensor according to claim 6, characterized in that specifically heavy metal ion-binding groups are selected from the group of proteins having the sequences SEQ.ID.NO.1, SEQ.ID.NO.2, SEQ.ID.NO.3 or SEQ.ID.NO.4, or functional derivatives thereof having equivalent binding characteristics.

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b) contacting said sensor with a reference liquid not containing said heavy metal ion of interest and determining the capacitance according to per se known methods;
c) contacting said sensor with a sample suspected of containing said heavy metal ion and determining the capacitance according to per se known methods; and
d) calculating the difference between the capacitance of the sample and the capacitance of the reference sample, and optionally calculating the amount of said compound by using prerecorded calibration data.

9. A method according to claim 8 for determining the presence of ions selected from the group of Zn^{2+}, Hg^{2+}, Cd^{2+}, Cu^{2+}, and Pb^{2+}.

10. Use of a sensor according to claim 6 for determining the presence of of ions selected from the group of Zn^{2+}, Hg^{2+}, Cd^{2+}, Cu^{2+} and Pb^{2+}.